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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Kazuyuki HAMADA, et al., Group Art Unit: 1638

Serial No.: 09/509,945

Filed: April 4, 2000

For: MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patent and Trademarks
Washington, D. C. 20231

Sir:

I, Kazuyuki Hamada, a Japanese citizen, residing at c/o Japan Tobacco Inc., Plant Inovation Center, Higashibara 700, Toyoda-cho, Iwata-gun, Shizuoka 438, Japan, hereby declare and state that I am one of the applicants of the above-entitled application.

I declare that I received a Master's Degree in Biochemical Regulation from the Faculty of Agricultural Sciences, Nagoya University in March, 1991 and have been employed by Japan Tobacco Inc., the assignee of this application, since April, 1991.

I also declare that my present position is a research scientist of the assignee.

I declare further that I have read all of the Official Actions in the above-entitled patent application, and have read, and am familiar with, each of the references cited in the Official

Actions by the Examiner.

I declare further that the following test was conducted at my direction and under my supervision and the test results are true and correct to the best of my knowledge.

Ability to induct frame shift of mutant barnase derived sequence in corn protoplast

[Materials and Methods]

I considered that a nucleotide sequence, which induces a frame shifting during the translation, is present in the mutant barnase gene between the nucleotide insertion site and the termination codon which appeared at 9th frame due to the insertion of T. Thus, a plasmid with additional 16 bp fragment having the mutation site and the adjacent termination codon as those in the mutant barnase gene following immediately to the initiation codon of luciferase gene in plasmid pDO432 (Ow, et al., 1986; Figure 1) was constructed (Table 1, pLC031). The expression of the luciferase in corn protoplast by the construct was compared with pDO432 and two other constructs shown in Table 2 (Figure 2).

The protoplast was prepared from corn etiolated leaf at 10 days after germination according to the method of Sheen et al. (1991), and was suspended in an electroporation buffer (0.6M mannitol, 20mM KCl in 4mM Mes-NaOH, pH 5.7). 30μg of the plasmid of testing, 30μg of pBI221 (Genbank acc.# AF502128), and 75μg of the salmon sperm DNA were mixed with 0.8 ml of the suspension containing 1×10^5 cells, then electroporation was performed (125 mF and 400 V/cm). After 16 hours standing in darkness at 25℃,

the mixture was re-suspended in 75µl of 50 mM Tris-H_3PO_4, pH 7.5. PicaGene Kit (Toyo Ink Mfg. Co., Ltd) was used for the measurement of the luciferase activity. 75µl of the lysis reagent attached to the kit was added to the cell suspension, followed by destruction of the cell with sonication and luciferase activity was measured according to the manufacturer's instruction. Further, the value of luciferase activity was corrected in terms of the relative plasmid introduction efficiency which was calculated from pBI221-derived GUS (β-glucuronidase) activity (Ow, et al.; 1986).

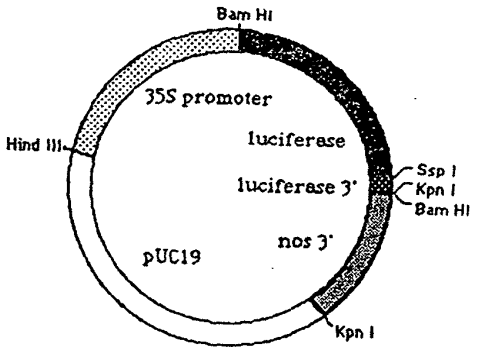

Figure 1. pDO432 (4549bp)

Table 1. Fragments inserted in the three pDO432-derived plasmids

Constructs	Sequences
pLC021	5'- atg GTT ATC AAC ACG TTT gaa - 3'
pLC031	- atg GTT ATT CAA CAC GTT TGAa -
pLC041	- TAA TTA ATC AAC ACG TTT gaa -
pDO432	- atg gaa -

Stop codon is underlined.

[Results and Discussion]

With pLC031, it was supposed that luciferase would not be

expressed unless the correct frame was to be restored by the frame shifting during the translation, because when the translation starts at the ATG, the termination codon appears at the 7th frame so that the translation stops at this site. However, by comparing with the controls (pDO432, pLC021), a weak but clear expression of luciferase was detected from the protoplast transformed with pLC031 (Figure 2). Since such activity was not detected in the case of the construct with the destructed initiation codon (pLC041), the luciferase expression of pLC031 would not have been attributable to irregular translation initiation. Therefore, the results support the hypothesis that the frame shifting during the translation, at a site inside of the 16 bp insertion sequence from the mutant barnase gene, restored the correct translation frame.

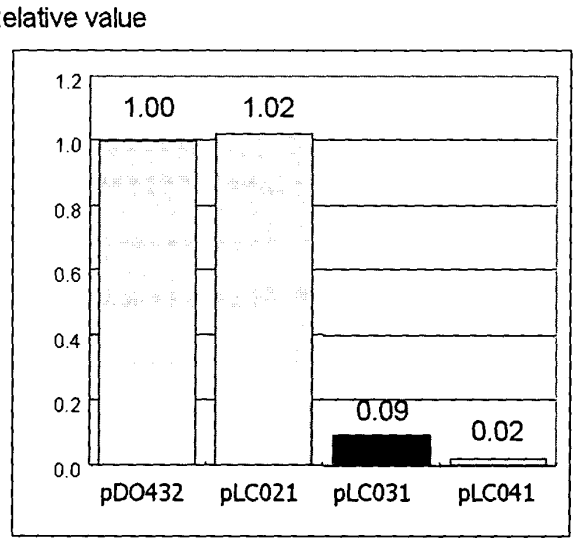

Figure 2. Luciferase activity expressed in maize protoplast

[References]

- Ow, D.W., Wood, K.V., DeLuca, M., De Wet, J.R., Helinski, D.R.,

and Howell, S.H., (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234, 856-859.

- Sheen, J., (1991). Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. Plant Cell 3, 225-245.

I declare further that all statements made therein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issuing thereon.

Dated this 30th day of October, 2002

Kazuyuki Hamada

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